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# NEONATAL HYPOXIC ISCHEMIC ENCEPHALOPATHY: INFLAMMATION AND THERAPIES

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# Neonatal hypoxic ischemic encephalopathy: inflammation and therapies

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

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*To my husband*

*and*

*To my family*

Dimidium facti qui coepit habet; sapere aude, incipe. [Horatius]

He who has begun is half done; dare to know, begin! [Horace]

# ABSTRACT

Neonatal hypoxic ischemia (HI) is a severe condition characterized by a complex pathophysiology. The lack of oxygen (hypoxia) and blood flow (ischemia) leads to neuronal cell death via necrosis and apoptosis, and a consequent post-ischemic inflammation. HI brain injury may lead to seizures, cognitive and motor impairments, and death. Worse neurodevelopmental outcomes have been observed in male than in female survivors, thus underlining sex-dependent differences.

To date, hypothermia is the only available evidence-based treatment for neonatal HI that shows neuroprotection if applied within six hours after the insult. Although hypothermia reduces production of cytokines and metabolic stress, it was shown to not be effective in severe cases of neonatal HI. Additional therapies meant to alleviate the HI outcomes are therefore needed.

In the present thesis we investigated the role of two key players involved in post-ischemic inflammation, namely resident microglia and infiltrating macrophages, and studied the effects of drug- and cell-based treatments aimed at reducing injury.

HI was induced by occluding the common carotid artery in mouse pups that were then subjected to hypoxia. By investigating the dynamics of inflammatory cells in the hippocampus of injured mice, sex-specific differences were observed in microglia and infiltrating peripheral-macrophages. Sequencing data revealed that macrophages are the drivers of the post-ischemic inflammation through significant upregulation of cytokine, chemokine and sensome markers, as well as activation genes. In addition, microglial cells, which were shown to downregulate unique signature genes upon inflammation, restored their homeostatic role within three days after injury in males, suggesting a different mechanistic effect in response to the neuroinflammatory cascade.

The role of resident microglia was further investigated in a Tamoxifen-based depletion model in which HI was then performed. While no difference in the speed of microglial repopulation was observed between males and females, the injury progression and cytokine production changed in a sex-dependent fashion. Specifically, depletion aggravated neuronal damage and apoptosis in male mice following HI.

In order to reduce inflammation and to induce neuroprotection, therapies involving caffeine or bone marrow-derived macrophage administration were assessed in this thesis. Caffeine, which is an adenosine-receptor competitor, is currently used in the clinic as treatment for neonatal apnea. As long-term follow-up studies of apneic babies treated with caffeine showed a reduced incident of cerebral palsy, this drug was administered at different time points after HI. Our results revealed a reduced lesion and improved behavioral outcomes after a single dose of 5 mg/kg caffeine immediately post-HI, with a reduction of the lesion and glial scar extent, and modulation of microglia activation and pro-inflammatory genes.

Bone marrow-derived macrophages were adoptively transferred 5 days after HI to investigate their immunomodulatory and wound healing properties. Our results showed a clear difference when anti-inflammatory macrophages (M2) or unpolarized control cells (M0) were administered. While M2 cell therapy led to functional recovery, we observed that M0 macrophages worsened behavioral outcomes and increased the injury size. In addition, *in vitro* studies in organotypic hippocampal slices co-cultured with these macrophages showed that, while M2 maintained memory of their phenotype, the M0 cells became polarized towards a pro-inflammatory state, thus suggesting how unpolarized cells could lead to exacerbation of the inflammation and the consequent worsening of injury extent and behavioral performance observed *in vivo*.

In summary, in this thesis I highlight the importance of microglia and infiltrating macrophages in the post-ischemic inflammatory cascade, and how caffeine and bone-marrow derived macrophages may be of potential therapeutic interest in future studies.

# LIST OF SCIENTIFIC PAPERS

I. **Elena Di Martino**, Takashi Umekawa, Anoop Ambikan, Sarantis Giatrellis, Daniel Ramsköld, Davide Vacondio, Ahmed Osman, Qiaolin Deng, Jonas Frisen, Rickard Sandberg, Ujjwal Neogi, Ulrika Ådén, Volker M. Lauschke, Klas Blomgren and Julianna Kele. *Sex-dependent and -independent inflammatory patterns in resident microglia and infiltrated macrophages after neonatal asphyxia.*
**Manuscript**

II. Shunichiro Tsuji, **Elena Di Martino**, Takeo Mukai, Shoko Tsuji, Takashi Murakami, Robert A. Harris, Klas Blomgren, Ulrika Åden. *Aggravated brain injury after neonatal hypoxic ischemia in microglia-depleted mice.*
**Journal of Neuroinflammation** 17(1):111 (April 2020)

III. **Elena Di Martino**, Erica Bocchetta, Shunichiro Tsuji, Takeo Mukai, Robert A. Harris, Klas Blomgren, and Ulrika Ådén. *Defining a time window for neuroprotection and glia modulation by caffeine after neonatal hypoxia-ischaemia.*
**Molecular Neurobiology** 57(5):2194-2205 (May 2020)

IV. **Elena Di Martino**, Davide Vacondio, Luigi Balasco, Luis Enrique Arroyo-Garcia, Takeo Mukai, Melanie Pieber, Anne-Kristin Kukla, Shunichiro Tsuji, André Fisahn, Xingmei Wang, Ronny Wickström, Klas Blomgren, Robert A. Harris and Ulrika Ådén. *Adoptive transfer of bone-marrow-derived macrophages modulates the post-ischemic inflammation in a model of neonatal hypoxia-ischemia.*
**Manuscript**

# PAPERS NOT INCLUDED IN THIS THESIS

I. Takeo Mukai, **Elena Di Martino**, Shunichiro Tsuji, Klas Blomgren, Tokiko Nagamura-Inoue, Ulrika Ådén. *Umbilical cord-derived mesenchymal stromal cells immunomodulate and restore actin dynamics and phagocytosis of LPS-activated microglia via PI3K/Akt/Rho GTPase pathway.*
**Submitted** to **Journal of Neuroscience Research.**

II. Giulia Zanni*, Shinobu Goto*, Adamantia F. Fragopoulou*, Giulia Gaudenzi[#], Vinogran Naidoo[#], **Elena Di Martino[#]**, Gabriel Levy, Cecilia A. Dominguez, Olga Dethlefsen, Angel Cedazo-Minguez, Paula Merino-Serrais, Antonios Stamatakis, Ola Hermanson, Klas Blomgren. *Lithium treatment reverses irradiation-induced changes in rodent neural progenitors and rescues cognition.*
**Molecular Psychiatry** 10.1038/s41380-019-0584-0 (November 2019)

III. Giulia Zanni, Wojciech Michno, **Elena Di Martino**, Anna Tjärnlund-Wolf, Jean Pettersson, Charlotte E. Mason, Gustaf Hellspong, Klas Blomgren, Jörg Hanrieder. *Lithium accumulates in neurogenic brain regions as revealed by high resolution ion imaging.*
**Scientific Reports** 18;7:40726 (January 2017)

IV. Giulia Zanni*, **Elena Di Martino***, Anna Omelyanenko, Michael Andäng, Ulla Delle, Kecke Elmroth, Klas Blomgren. *Lithium increases proliferation of hippocampal neural stem/progenitor cells and rescues irradiation-induced cell cycle arrest in vitro.*
**Oncotarget** 10;6(35):37083-97 (November 2015)

* Shared first author
[#] Shared second author

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| ARs | Adenosine receptors |
| ATP | Adenosine triphosphate |
| BBB | Blood-brain barrier |
| BMDMs | Bone marrow derived monocytes |
| CCL2 | Chemokine (C-C motif) ligand 2 |
| CCR2 | C-C chemokine receptor type 2 |
| CNS | Central nervous system |
| CP | Cerebral palsy |
| CSF | Cerebrospinal fluid |
| CSF1R | Colony stimulating factor 1 receptor |
| CX3CR1 | CX3C chemokine receptor 1 |
| DAB | 3,3′-Diaminobenzidine |
| DAMP | Damage-associated molecular pattern |
| DT | Diphtheria toxin |
| EYFP | Enhanced yellow fluorescence protein |
| FACS | Fluorescence-activated cell sorting |
| GFAP | Glial fibrillary acidic protein |
| GFP | Green fluorescent protein |
| GW | Gestational week |
| HI | Hypoxia/ischemia |
| HIE | Hypoxic ischemic encephalopathy |
| Iba1 | Ionized calcium binding adaptor molecule 1 |
| IL | Interleukin |
| iNOS | Induced nitric oxide synthase |
| i.p. | Intraperitoneal |
| LPS | Lipopolysaccharide |
| MAP2 | Microtubule-associated protein 2 |
| M-CSF | Macrophage colony stimulating factor |
| MBP | Myelin basic protein |
| MCP-1 | Monocyte chemoattractant protein 1 |
| MMP | Matrix metalloproteinases |
| NMDA | N-methyl-D-aspartate |
| PAMP | Pathogen-associated molecular pattern |
| PFA | Paraformaldehyde |
| PND | Postnatal day |
| PRR | Pattern-recognition receptor |
| RFP | Red fluorescent protein |
| RNA | Ribose nucleic acid |

| | |
|---|---|
| ROS | Reactive oxygen species |
| RT-qPCR | Reverse transcription quantitative polymerase chain reaction |
| TGF-β | Transforming growth factor beta |
| TH | Therapeutic hypothermia |
| TLR | Toll-like receptor |
| TNFα | Tumor necrosis factor alpha |
| UCBCs | Umbilical cord blood cells |
| WT | Wild type |

# 1 INTRODUCTION

## 1.1 BRAIN DEVELOPMENT/CLINICAL BACKGROUND

In humans, brain developments starts when the neural plate emerges from the neuroepithelial cells of the ectoderm during the third gestational week (GW3) (Stiles & Jernigan, 2010). In GW5, progenitor cells such as radial glial cells start dividing and forming brain vasculature (Budday, Steinmann, & Kuhl, 2015). Synaptogenesis begins as early as GW8 (Molliver, Kostovic, & van der Loos, 1973) as a consequence of neurogenesis that peaks between GW8-14 (Clancy et al., 2007) and continues through early postnatal life (Sanai et al., 2011). As development proceeds, other glial cells are generated. Astrocytes appear in GW15 (Roessmann & Gambetti, 1986), while oligodendrocyte (OL) progenitor cells appear in GW17 and then differentiate into mature OL up to early childhood (Rakic & Zecevic, 2003; Yeung et al., 2014). A fourth and ontogenetically distinct cell type, the microglia, appears in the human fetal brain approximately in GW5 and originates from yolk sac primitive macrophages (Ginhoux et al., 2010; Kierdorf et al., 2013) although properly differentiated microglia are not observed until GW35 (Verney, Monier, Fallet-Bianco, & Gressens, 2010). These complex processes of cell genesis, maturation and organization that shape the developing brain continue untill post-natal life (Giedd et al., 1999). The perinatal period is thus a critical time for brain development, and injuries at this timepoint could irreperably alter the normal development and maturation of different cell populations.

### 1.1.1 Perinatal brain injury

Estimates from 2015 indicate that the global under 5-year-old mortality rate is 43 per 1000 live births, of which 45% of the deaths are during the neonatal period (Victora et al., 2016). Neonates are at high risk of perinatal brain injuries and epidemiological studies have identified intrapartum complications and perinatal infection as risk factors (Dammann & Leviton, 1997). Injuries during the perinatal period include systemic inflammation, preterm brain injury, perinatal asphyxia and stroke (Hagberg, David Edwards, & Groenendaal, 2016). It is notable that independently of the cause that leads to brain injury in term and preterm infants, post-injury inflammation is a common feature (Hagberg et al., 2016).

### 1.1.2 Hypoxic-ischemic brain injury

Hypoxic-ischemic (HI) brain injury is a severe condition characterized by a complex pathophysiology. It is not dependent on a single event, but rather consists of a series of pathological responses. The primary cause is an impaired blood flow and the consequent dysfunction of one or more organs, typically the brain due to its high demand of oxygen and nutrient supply (Martin-Ancel et al., 1995; Volpe, 2001). The incidence of a moderate to severe HI for term babies accounts for 0.5-2 per 1000 live births in developed countries (Kurinczuk, White-Koning, & Badawi, 2010), but the number is even higher in low income settings (Costello & Manandhar, 1994).

Term neonates that develop hypoxic ischemic encephalopathy (HIE) display symptoms such as seizures, feeding and breeding difficulties, altered tonus and often present a low

APGAR score associated with umbilical cord acidosis (K. A. Allen & Brandon, 2011; Pierrat et al., 2005).
HIE can lead to serious neurological impairments that affects motor and cognitive functions, and life-long consequences that range from mental retardation to cerebral palsy (CP), the latter which is the leading cause of motor disability in children (Volpe, 2001).

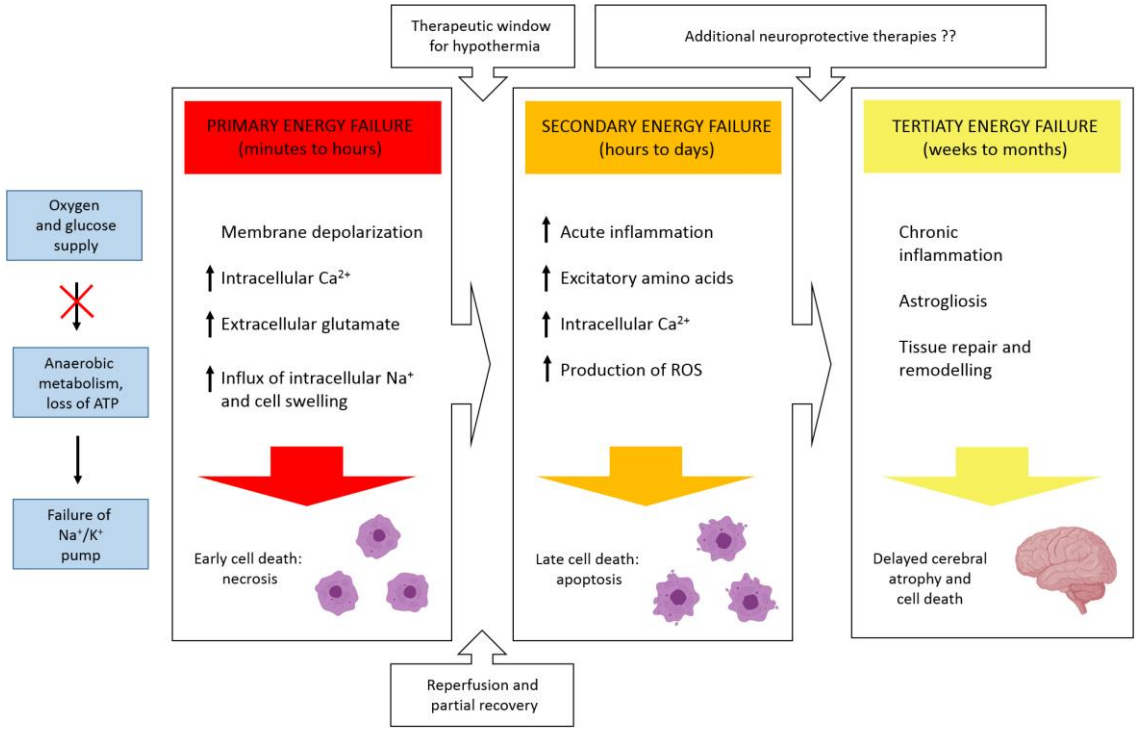



Figure 1: Mechanism of HI brain injury. Lack of oxygen and glucose leads to a primary energy failure that causes an early cell death via necrosis. The brain reperfusion leads to a partial recovery of oxidative metabolism that causes a secondary energy failure and cell death via apoptosis. Long-term inflammation and astrogliosis define the tertiary phase that leads to delayed cell death. Image adapted from Li et al. (B. Li, Concepcion, Meng, & Zhang, 2017) and partially created with BioRender.

### 1.1.3 Mechanisms of hypoxic-ischemic brain injury

The collapse of blood and oxygen supply, termed ‘primary energy failure’, starts a cascade of events that lead to a delayed metabolic disruption referred to as ‘secondary energy failure’. The time between the primary and the secondary phases, the ‘latent phase’, allows a brief period of recovery and it varies in length ranging from hours to days (Ten & Starkov, 2012). It has been suggested that there is a ‘tertiary phase’ during which possible factors such as glial scar, persistent inflammation and epigenetic changes cause long-lasting effects and promote further damage (Fleiss & Gressens, 2012). This three-phase mechanism is summarised in Figure 1.

Initially, the lack of energy caused by HI inhibits mechanisms that act to maintain cellular homeostasis and lead to the failure of the $Na^+/K^+$ pump and the consequent $Na^+$, $Cl^-$ and $H_2O$ influx (Choi, 1988). The resulting cell edema causes neuronal depolarization and the excessive release of glutamate, an excitatory neurotransmitter that under physiological conditions is usually cleared by astrocytes (Hagberg et al., 2016). The accumulation of glutamate in the synaptic cleft, stimulates the N-methyl-D-aspartate (NMDA) receptors which massively

release intracellular $Ca^{2+}$ ions that activate the apoptotic cell death pathway and induce the production of reactive oxygen species (ROS) with ensuing mitochondrial damage (Hagberg, Mallard, Rousset, & Thornton, 2014). HI-induced excitotoxicity and oxidative damage cascades can instigate microvascular injury and blood-brain barrier (BBB) dysfunction, triggering a robust post-ischemic inflammation, as evidenced by microglia activation and cytokine release (Bona et al., 1999; Mallard, Tremblay, & Vexler, 2019; McRae, Gilland, Bona, & Hagberg, 1995). The role of inflammation, together with necrosis and apoptosis, is of critical importance in the secondary and especially tertiary phases of brain injury, suggesting a potential target for therapeutic intervention (Hagberg et al., 2015).

## 1.2 NEURO-INFLAMMATION

Inflammation is common to various forms of central nervous system (CNS) injuries and diseases, although is not necessarily a causative factor. Inflammation is characterized by the activation of different cell types including microglia and astrocytes and the production of inflammatory molecules. It is increasingly considered to contribute to the process of pathogenesis and, where appropriate, to repair (Hagberg, Gressens, & Mallard, 2012; Hagberg et al., 2015). In the context of neonatal HI, inflammation is critical for delayed cell death and contributes importantly to the progression of brain injury (Inder & Volpe, 2000; Mallard, Ek, & Vexler, 2018). In addition, it has been widely established that due to the release of inflammatory molecules and the disruption of the BBB after HI, peripheral immune cells can infiltrate the brain in support of local microglia (Mallard et al., 2018; Mallard et al., 2019; P. L. P. Smith et al., 2018; Winerdal et al., 2012). This process is summarized in Figure 2.

### 1.2.1 CNS immune specialization

Tissue swelling and accumulation of cells at the inflammatory site - which is common in peripheral inflammation - is not well tolerated in the brain due to the dangerous level of pressure that may arise within the skull (Callahan & Ransohoff, 2004). In addition, the delicate neuronal circuitry development and the limited capacity for regeneration make the brain particularly sensitive, thus underlining the importance of maintaining homeostatic CNS functions (Carson, Doose, Melchior, Schmid, & Ploix, 2006).

The brain has been considered as an immune privileged organ for many years, relatively isolated from the periphery due to the existence of the BBB (Schwartz, Moalem, Leibowitz-Amit, & Cohen, 1999) that strictly regulates the ionic balance and prevents toxins and proteins from the blood affecting the CNS (Abbott, 2013). This immune privilege, although constrained to the cerebral parenchyma and not the ventricles, meninges and choroid plexus (Galea, Bechmann, & Perry, 2007), was later reconsidered after a functional network of lymphatic vessels lying parallel to the dural synuses were discovered (Louveau et al., 2015).

The isolation of the CNS is undermined during pathological conditions, being they infectious or traumatic, as neuroinflammation and the alteration of the BBB functionality attract peripheral immune cells (Mallard et al., 2018; Rayasam, Faustino, Lecuyer, & Vexler, 2020; Schafer et al., 2012). In the context of perinatal HI, inflammation and its key players are important contributors to both injury outcomes and an altered development of the immature brain (Hagberg et al., 2015).

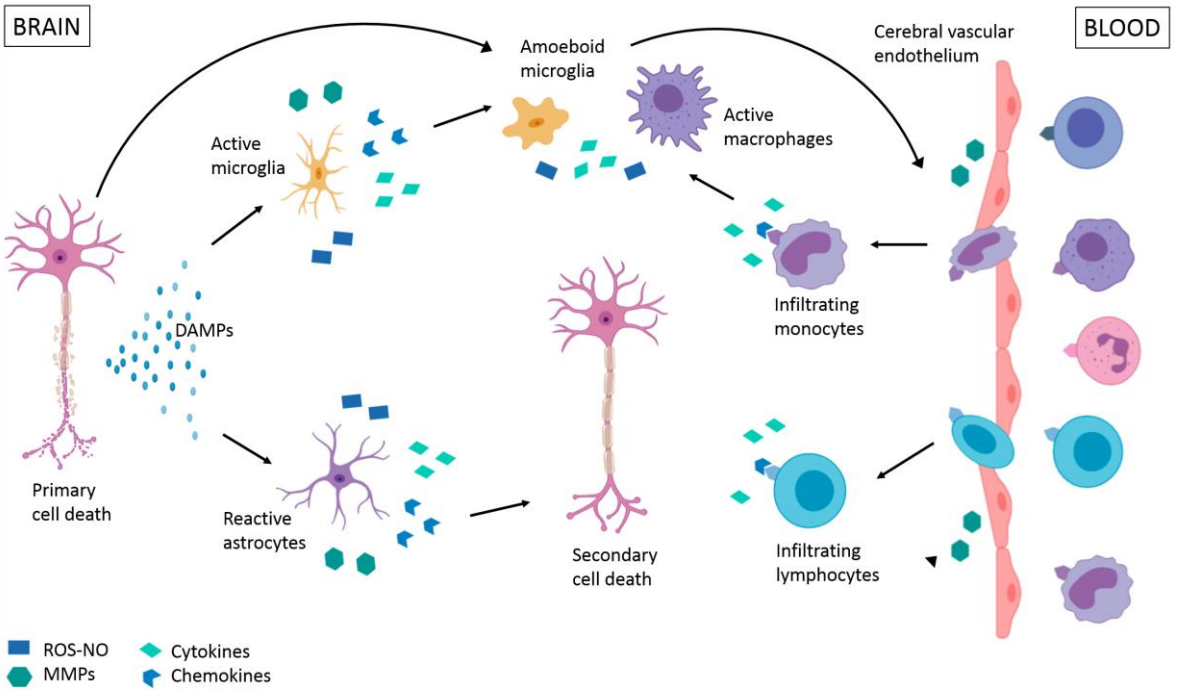



Figure 2: Post-ischemic inflammation. Primary neuronal death via necrosis leads to the release of DAMPs that are sensed by resident immune cells effectors. The latter start the inflammatory cascade by producing immunomodulators and ROS, resulting in a neurotoxic environment that promotes secondary cell death via apoptosis. In addition, the production of MMPs and chemokines induces BBB opening and the consequent infiltration of peripheral immune cells that exacerbate the inflammation and subsequent neuronal death. Image adapted from Li et al. (B. Li et al., 2017) and created with BioRender.

### 1.2.2 Resident microglia

Microglia are a primary immune cell in the brain and represent 12-15% of the CNS cellular component (Lawson, Perry, & Gordon, 1992). Microglia contribute to immune responses against infectious agents, degenerative diseases, trauma, they provide support for CNS development, myelination and synaptogenesis, and sustain brain homeostasis and structure (Casano & Peri, 2015; Nguyen et al., 2020). Microglia are a unique cell population and can be distinguished from other brain cells by origin, function, morphology and gene expression patterns (Nayak, Roth, & McGavern, 2014). Microglia derive from the yolk sac and migrate into the murine brain during an early embryonic stage before BBB closure (Alliot, Godin, & Pessac, 1999; Alliot, Lecain, Grima, & Pessac, 1991; Ginhoux et al., 2010).

Microglia enter the brain in an amoeboid state due to their high proliferation and phagocytic activity, which are behaviors also recognized during inflammatory processes (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011). During the early phases of development, microglia are highly motile to compensate for their low density in the brain parenchyma (Smolders et al., 2017). Colonization follows a dynamic spatial pattern, with initial accumulation in areas with proliferating neural precursors and developing axons, only to leave and finally distribute when neuronal functional structures and wiring connections have been established (Gordon & Pluddemann, 2017). Microglial functions in the developing brain thus specifically range between the elimination of apoptotic cells and the support of neurogenesis and angiogenesis, to the contribution of the generation of new astrocytes and oligodendrocytes (Frost & Schafer, 2016). Little is known about early microglia colonization and maturation in

the human brain (Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013; A. M. Smith & Dragunow, 2014) because data have mainly been acquired from animal studies.

Through a series of transitions the microglial population subsequently acquires its ramified morphology and reaches the mature phenotype by the end of the second postnatal week (Bennett et al., 2016; Matcovitch-Natan et al., 2016). In the adult brain microglia are distributed throughout the parenchyma with region-specific densities and diversity (Grabert et al., 2016; Lawson, Perry, Dri, & Gordon, 1990; Silvin & Ginhoux, 2018). After initial expansion via proliferation, the population of microglial cells remains relatively stable throughout life and is maintained by self-renewal (N. J. Allen & Barres, 2009; Harry, 2013). Microglia indeed have a long life span and proliferate to replace dying microglial cells, thus maintaining a steady-state condition (Ajami, Bennett, Krieger, Tetzlaff, & Rossi, 2007; Askew et al., 2017).

Although in physiological conditions microglia are considered to be in a resting state, they monitor, scan and control the nervous tissue, building a structured network of dynamic and plastic cells (Salter & Beggs, 2014). They constantly move the ramified processes to survey and check the surroundings, sensing microenvironmental alterations (Nimmerjahn, Kirchhoff, & Helmchen, 2005; Tay, Savage, Hui, Bisht, & Tremblay, 2017). Under pathological conditions microglia undergo rapid morphological changes: they retract their long processes and adopt an amoeboid shape, altering their genetic profile to adapt to new functions and conditions (Hanisch, 2002; Hirbec et al., 2018). During this process, which is called 'activation', microglia can rearrange and upregulate the surface receptors needed for changes in intracellular enzyme production, interaction with extracellular matrix and neighboring cells, and they release inflammatory and immunomodulatory molecules (Kettenmann et al., 2011; Nayak et al., 2014). Among all factors that microglia release, there are also chemoattractants such as monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) that recruit other immune cells such as macrophages from the periphery (Diab et al., 1999; Spanaus et al., 1997).

### 1.2.3 Infiltrating macrophages

Monocytes are innate immune cells of myeloid origin. They derive from bone-marrow hematopoietic stem cells, which differentiate into common myeloid progenitors under the regulation of macrophage-colony stimulating growth factor (M-CSF) (Cecchini et al., 1994; Gordon & Pluddemann, 2017). They are released into the blood circulation after maturation due to the chemokine C-C motif ligand 2 (CCL2), a chemokine also involved in macrophage attraction during pathological inflammatory conditions (Serbina & Pamer, 2006).
Monocytes can be divided into two main groups: classical ($Ly6^{hi}CCR2^{+}CX3CR1^{mid}$) and non-classical ($Ly6^{low}CCR2^{-}CX3CR1^{hi}$)(Geissmann, Jung, & Littman, 2003). The first are considered highly mobile but short-lived cells that can reach the site of injury and respond to inflammation (Auffray et al., 2007). The function of non-classical monocytes, which are thought to derive from the classical ones, is not yet well established, but they have longer half-life and patrol the vessel lumen in support of tissue homeostasis and repair (Ginhoux & Jung, 2014; Yona et al., 2013).

Monocytes are particularly important during inflammatory processes because they are involved in the production of monocyte-derived macrophages (MDMs) characterized by the expression of high levels of CD45 and are positive for CD11b (Davies, Jenkins, Allen, & Taylor, 2013; Epelman, Lavine, & Randolph, 2014; Ginhoux & Jung, 2014). MDMs are plastic

cells and have the function to survey, sense and scan the surrounding environment looking for pathogens. Under pathological conditions, be they infections or sterile injuries, they are recruited via specific signaling in the affected area and assist resident macrophages in the clean-up processes, thereby promoting a return to homeostasis (Murray & Wynn, 2011; Shi & Pamer, 2011).

In the case of HI these bone marrow-derived monocytes are recruited from the circulation into the CNS where they differentiate into macrophages to participate in the inflammatory response (Hellstrom Erkenstam et al., 2016; P. L. P. Smith et al., 2018; Umekawa, Osman, Han, Ikeda, & Blomgren, 2015). Although discrimination of resident microglia and infiltrating MDMs is quite difficult due to the similarity of these two cell types (e.g. surface marker expression (Perry, Hume, & Gordon, 1985; Sedgwick et al., 1991)), differences in their respective roles have been reported (Katsumoto, Lu, Miranda, & Ransohoff, 2014; Yamasaki et al., 2014). The specific interplay between these two cell types during pathological conditions, and extravasation of MDMs in the CNS, is therefore still a matter of study.

### 1.2.4 Phenotypes, function and nomenclature

Both macrophages and microglia are sentinels in their specific environments and have similar functions (Amici, Dong, & Guerau-de-Arellano, 2017). They have the capacity to recognize injured or dead cells and immune mediators, to produce small signaling molecules and to modulate/resolve inflammation (Herz, Filiano, Smith, Yogev, & Kipnis, 2017). They are specialized immunocompetent cells that express a wide range of receptors on their surface. The most important receptors for acute inflammation are the pattern recognition receptors (PRRs), which are divided into many subfamilies depending on their function. Activation of PRRs by ligation with pathogen- or danger-associated molecular patterns (PAMPs or DAMPs) leads to various intracellular pathways that result for example in the production of signaling mediators, phagocytosis and migration (Bianchi, 2007). A family of PRRs receptors that plays a key role in the activation of microglia/macrophages is the Toll-like receptors (TLRs). To date, 13 different receptors have been identified in mammals and they can be separated into subgroups depending on whether they are expressed on the cell surface or within intracellular vesicles. Although in the brain TLRs are mostly expressed by microglia, following ischemia/reperfusion injury TLR4 and TLR2 are also expressed by cortical neurons (Tang et al., 2007).

Depending on the surrounding cues and stimuli, microglia and macrophages can progressively polarize into specific activation states (Gordon & Martinez, 2010; Hanisch & Kettenmann, 2007; Harris, 2014; Lavin et al., 2014). Over time, microglial nomenclature has been associated with the general classification suggested to describe the ‘macrophages polarization paradigm’ inspired by Mills and colleagues (Mills, Kincaid, Alt, Heilman, & Hill, 2000). They described two distinct phenotypes: M1 (pro-inflammatory or classically activated macrophages) and M2 (anti-inflammatory or alternatively activated macrophages). The former are usually associated with the production of inflammatory molecules such as IL-1β, tumor necrosis factor-α (TNF- α) and nitric oxide (NO) following stimulation with lipopolysaccharide (LPS) or interferon-γ (IFN- γ), and are highly phagocytic. The M2 phenotype is instead associated with the production of factors that promote tissue remodeling such as IL-10 and arginase-1 following stimulation with IL-4 or IL-13. However, recent investigations have proven that this M1/M2 model does not reflect the real activation and polarization occurring *in vivo*, where the number of stimuli that these cells are exposed to are not limited to those

mentioned above and extensively used *in vitro* (Ginhoux, Schultze, Murray, Ochando, & Biswas, 2016; Ransohoff, 2016).

### 1.2.5 Signalling molecules

There is a variety of signaling molecules involved in the chain of events following an insult such as HI, and cytokines, adhesion molecules and matrix metalloproteases (MMPs) are the key components involved in the inflammatory process (Nissinen & Kahari, 2014; Turner, Nedjai, Hurst, & Pennington, 2014).

Cytokines, which are small signaling proteins of approximately 5-20 kDa, are of particular importance in the development of HI-related outcomes. They are subgrouped in families depending on their receptor type, for example interleukins (IL), interferons (IFN) and the tumor necrosis factor family (TNF) (Dinarello, 2007). They are produced by different brain cells as well as leukocytes - including macrophages and monocytes - but also by epithelial cells and fibroblasts, and act as modulating agents during inflammatory processes (Kennedy & Silver, 2016). Cytokines mediate a variety of functions by binding specific receptors and signaling in an autocrine, paracrine or endocrine manner (O'Shea, Gadina, & Siegel, 2013). For instance, they can modulate inflammation by promoting (pro-inflammatory) or hampering (anti-inflammatory) the response (Turner et al., 2014).

In HI, levels of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α are relatively high compared to controls as early as a few hours after HI, indicating the beginning of post-ischemic neuroinflammation. The concentration of these cytokines in the blood and cerebral spinal fluid (CSF) of asphyxiated babies was reported to be positively correlated with the severity of brain injury, therefore underlining the importance of these molecules to define the extent of the HI insult (Aly, Khashaba, El-Ayouty, El-Sayed, & Hasanein, 2006). However, such pro-inflammatory effects are antagonized by anti-inflammatory cytokines such as IL-10, which appears in the serum and brain of neonatal rats 6h and peaks at 48h after injury, and is suggested to resolve the inflammation by modulating the functions of leukocytes and glial cells (S. J. Li et al., 2014).

Chemokines are a separate subset of cytokines whose function is explained by their name: chemotactic cytokines. Their role is indeed in the recruitment of leukocytes to the site of inflammation. To facilitate the identification of chemokines, a subdivision in four families has been applied depending on where the N-terminal cysteine resides: CXC, C-C, C and CX3C (Murphy et al., 2000). Chemokines are less specific than other cytokines, and indeed many of the more than 50 ligands can bind to either one or several of the 23 receptors identified to date, and a receptor can have more than one specific ligand (Griffith, Sokol, & Luster, 2014). In the specific context of asphyxiated newborn babies, high levels of CXCL8 in the CSF are associated with the HIE severity and poor neurological outcomes (Savman, Blennow, Gustafson, Tarkowski, & Hagberg, 1998). CCL2, also known as MCP-1, is considered among the most important chemokines in HI. It is significantly upregulated in microglia and highly associated with the infiltration of macrophages and neutrophils at the injury site (Faustino et al., 2011; Galasso et al., 2000), as well as CCL7, CXCL1, CCR1 and CCR5, which were also observed to be upregulated after HI (Hedtjarn, Mallard, Eklind, Gustafson-Brywe, & Hagberg, 2004)

There are other signaling molecules that do not belong to the cytokine family, and they are MMPs and ADAMs (A disintegrin and A metalloproteases), or soluble adhesion molecules.

MMPs are zinc-dependent enzymes important in the maintenance of the function and integrity of physical barriers, as well as in the regulation of inflammatory mediators. They are classified depending on their structure and cellular localization (Nissinen & Kahari, 2014; Vandenbroucke & Libert, 2014). ADAMs are proteases similar to MMPs due to the zinc-dependent activity, and their role is to support the formation of adhesion molecules (Weber & Saftig, 2012). The latter are small molecules that exist in soluble isoforms and are important for intercellular communication and cell-to-cell contact. They are divided into integrins, selectins (E-, P- and L-selectins), and immunoglobulin family members (e.g. VCAM, ICAM). HI upregulates the expression of many adhesion molecules, especially VCAM-1 and ICAM-1, that have been suggested to play a critical role in monocyte trafficking and recruitment within the brain, thus playing a fundamental role in the injury cascade (Hedtjarn et al., 2004; Lai et al., 2017; Zonneveld et al., 2014).

## 1.3 THERAPIES

Perinatal asphyxia is an important social burden and to date several studies have been conducted in order to assess different approaches to treat HIE.

As previously described, HI involves an ongoing injury process in which the damage and the severity are connected to the duration of this process and its extent. In order to achieve a neuroprotective effect it is thus necessary to interrupt this chain of events. Although the interval between the primary and the secondary energy failure, called the latent phase, was suggested to represent an optimal therapeutic window for clinical intervention, the correct identification of the resolution of the primary energy failure and the beginning of this latency period in newborn children is difficult to establish (Cotten & Shankaran, 2010).

Encouraging data from preclinical studies in perinatal animal models have demonstrated that therapies applied during the latent phase lead to a successful reduction in brain damage, possibly by modulating the secondary energy failure phase (K. A. Allen & Brandon, 2011). However, only one successful therapy that is safely applicable in the clinic has been developed thus far.

### 1.3.1 Hypothermia

Hypothermia, which is currently the only available therapy to ameliorate HIE outcomes, consists of the progressive reduction of body (or head) temperature down to approximately 33°C within a 6h-window after the birth of an asphyxiated term baby, for a maximum of 72h (Perlman et al., 2015). By doing so the brain metabolism is reduced by approximately 5% for each 1°C decrease in body temperature, delaying the onset of anoxic cell depolarization, intracellular calcium influx, and the consequent glutamate and aspartate release (Laptook, Corbett, Sterett, Garcia, & Tollefsbol, 1995). Additionally, recent studies reported that hypothermia is involved in the modulation of inflammatory pathways by reducing proinflammatory cytokines, and attenuating microglia proliferation and neurotoxicity (Orrock et al., 2015; Seo et al., 2012; Sheth, Brito, Mukherjea, Rybak, & Ramkumar, 2014).

Clinical trials have indeed demonstrated that hypothermia treatment in newborn babies with moderate HIE reduced neurological sequelae and long-term prognosis (Shankaran, 2012), even though approximately half of them still displayed major neurological disabilities (Azzopardi et al., 2014). Although many studies have shown the positive effects of

hypothermia, this treatment is not free from side-effects, including pulmonary hypertension, coagulopathies and separation from the mother (Jacobs et al., 2013). In addition, the tight time-window of application and the need for intensive care indicate the need for additional treatments for hypoxic ischemic brain injury in neonates.

### 1.3.2 Caffeine

Caffeine is the most commonly used psychoactive drug worldwide (EFSA Panel on Dietetic Products & Allergies, 2015). It is a well-known methylxanthine whose hydrophobic properties allow it pass through all biological membranes, including the BBB, and guarantee an easy penetration of the CNS (Lachance, Marlowe, & Waddell, 1983; Tanaka, Nakazawa, Arima, & Iwasaki, 1984). Compared to other methylxanthines, caffeine is more easily absorbed, has a wider therapeutic range and longer half-life, and so it is preferably used in the clinic (Bertil B Fredholm, 2010).

Specifically, caffeine is a non-specific adenosine receptor (AR) antagonist and binds to some extent to three of the four ARs, denoted as adenosine $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ receptors, with a preference for $A_1$ and $A_{2A}$ in normal physiological conditions (B. B. Fredholm, AP, Jacobson, Linden, & Muller, 2011). In the brain these last two receptors are expressed in the pre- and post-synaptic sites of neurons, but are also present in other cells such as oligodendrocytes, astrocytes and of particular interest for this thesis, in microglia (Sheth et al., 2014). Caffeine is safely used in the clinic to treat birth apnea (Abu-Shaweesh & Martin, 2017) and follow-up studies have shown a decrease in CP and cognitive delay incidence at 18 months of age (Schmidt et al., 2007). Moreover, pre-clinical studies have reported a neuroprotective effect of caffeine in experimental models of neonatal asphyxia (Bona, Aden, Fredholm, & Hagberg, 1995). Caffeine was additionally proven to reduce: LPS-induced brain inflammation in adult rats (Brothers, Marchalant, & Wenk, 2010); TNF-a production in cord blood monocytes stimulated with LPS (Chavez-Valdez, Ahlawat, Wills-Karp, & Gauda, 2016); and TNF-a, PGE2 and NO in BV2 microglia exposure to LPS *in vitro* (Kang et al., 2012).

Despite the beneficial effects that caffeine appears to have, a series of studies have suggested that an inappropriate dose or treatment duration may have detrimental effects in the developing brain and its functions (Atik et al., 2017). If administered too early and for a long period of time, caffeine reduced myelin synthesis in newborn rats in a dose-dependent manner (Fuller & Wiggins, 1981). Similarly, another study reported reduced proliferation of neural stem cells and decreased formation of astrocytes in newborn mice as result of caffeine treatment (Desfrere, Olivier, Schwendimann, Verney, & Gressens, 2007). Hence, the appropriate combination dose/duration/timing of the treatment has yet to be found.

### 1.3.3 Cell-based treatments

Cell-based therapies have recently drawn attention due to their encouraging results in experimental studies both at clinical and animal levels, suggesting their potential application in preventing or modulating perinatal brain injuries.

The most frequently used cell types in these studies are mesenchymal stem cells (MSCs) from various tissue origins, umbilical cord blood cells (UCBCs) and, recently, also amniotic epithelial cells (AECs): all tissues that allow autologous administration reducing the chances of rejection. Additional pros of these cell types are the minimal *ex vivo* manipulation and storage properties, the availability at birth, and the non-invasive techniques to collect them

(being the cells obtained from tissues that would otherwise be discarded) (Castillo-Melendez, Yawno, Jenkin, & Miller, 2013).

The mechanisms of action of these cells seem to include multiple aspects. They are all immunomodulatory and have anti-inflammatory properties, reduce apoptosis and oxidative stress and enhance the regenerative process by secreting various factors and promote angiogenesis and neurogenesis (Glenn & Whartenby, 2014; Nabetani, Shintaku, & Hamazaki, 2018). UCBCs were indeed shown to: inhibit microglial activation following HIE (Pimentel-Coelho et al., 2009); to attenuate reactive gliosis and reduce the infiltration of leukocytes into the brain by restoring the BBB (Wasielewski, Jensen, Roth-Härer, Dermietzel, & Meier, 2012); and were proven to reduce production of TNF-α and increase levels of IL-10 in the spleen of rats with HI (Vendrame et al., 2006). Administration of UCBCs in animal studies of HIE has also resulted in functional recovery such as improved sensorimotor reflexes and motor performance, and had neuroprotective effects if given intraperitoneally (i.p) soon after the lesion (Meier et al., 2006; Pimentel-Coelho et al., 2009). Interestingly, later treatments were also reported to be beneficial with intravenous (i.v) administration of UCBCs a week after HI. However, other studies reported controversial results, stating the lack of beneficial effects of UCBCs i.v if given 24 or 48h after injury (de Paula et al., 2009; Ohshima et al., 2016).

For MSCs, an increase of OL progenitors, mature OL and myelin formation in the ischemic hemisphere were all observed in the pioneering study of van Velthoven and colleagues (van Velthoven, Kavelaars, van Bel, & Heijnen, 2010). A recent study showed that P7 rats receiving MSCs i.v three days after HI made less mistakes in the beam walking test, had increased brain volume and enhanced synaptogenesis (Sakai et al., 2018). Additionally, it was observed that P9 mice pups receiving MSCs intranasally after HI had improved object recognition and barn maze test perormance, preserved white matter, reduced leukocyte infiltration and microglial downregulation (Herz et al., 2018). Similarly, MSCs were also protective when administered 10 days after HI, reducing the number of activated microglia and changing their polarization towards an anti-inflammatory phenotype, and limiting the astrocytic scar near the lesion (Donega et al., 2014).

In the last decade there has been a growing interest in human AECs and their reparative properties. Recent data in a neonatal mouse model of systemic inflammation combined with hyperoxia has shown a reduction of apoptosis and astrocyte areal coverage after treatment with hAECs, and increased microglia density and activation. However, when co-cultured *in vitro* with LPS-stimulated microglia, hAECs increased microglial phagocytic activity and decreased their pro-inflammatory phenotype (Leaw et al., 2017). Additionally, in a model of pulmonary inflammation in preterm lambs, hAECs reduced pro-inflammatory cytokines and macrophage presence in the lungs (Melville et al., 2017), suggesting a similar immunomodulatory effect in multiple organs.

## 1.4 SEX DIFFERENCES

It has long been acknowledged that newborn boys have a higher risk for complications during the neonatal period than do girls, and that they also are more prone to present with neurodevelopmental impairments (Wood et al., 2005). Infants with HI injury show impairments in language/speech and visual attention, deficits in learning, memory and executive functioning, display poor performance on spatial memory tasks and have lower IQ

scores. Among these, male neonates are reported to have worse outcomes compared to females with similar injury (Aylward, 2002).

Similarly, sex-specific impairments have been also observed in behavioral outcomes of experimental animals subjected to HI. As recently summarized by Netto and colleagues, female rats showed greater memory deficits, while males instead had greater motor impairments (Netto, Sanches, Odorcyk, Duran-Carabali, & Weis, 2017). Although the nature of the specific mechanisms explaining this difference between males and females remains unclear, recent studies have identified several biological processes known to play key roles in the long-term outcomes. In fact, data from preclinical studies show also that: 1) male animals are more susceptible to oxidative stress; 2) cell death is mainly dependent on caspase activation in female rats; and 3) the microglial response in the male brain is more pronounced during the chronic phase of inflammation compared to in females (Charriaut-Marlangue, Besson, & Baud, 2017). In particular, microglia are reported to have a sexual dimorphisms in the neonatal brain in physiological conditions, with differences evident in microglial cell shape and density over time and in different brain regions (Lenz & McCarthy, 2015; Schwarz, Sholar, & Bilbo, 2012). Considering the evidence supporting sexual dimorphism in this injury model, we kept this parameter into account when analysing the data included in this thesis.

# 2 AIMS

### General aim

The main aim of this thesis was to characterize post-ischemic neuroinflammation and to explore therapeutic targets after HI brain injury.

### Specific aims

1. to characterize resident microglia and infiltrating macrophages in the hippocampus after HI, and to define possible sex-specific differences.

2. to study the role of microglia and effects that its depletion has on HI brain injury in a model of tamoxifen-based microglia-depletion.

3. to investigate whether caffeine exerted neuroprotection after HI, and to define the therapeutic time-window.

4. to study the effects of adoptive transfer of bone marrow-derived macrophages in different polarization states after HI.

# 3 METHODS

A detailed methodology description is provided in each manuscript. In this section I will therefore focus on ethical considerations and I briefly describe the *in vivo* and *in vitro* models of injury, and the main techniques.

## 3.1 ETHICAL CONSIDERATIONS

All studies in this thesis were approved by Swedish Ethical Committee for Animal Research, Stockholms Norra Djurförsöksetiska Nämnd, and following local and regional guidelines and the Directives N249/13, N94/15 and N126/16.

All mice were pathogen-free and were bred in house. The dams and pups had free access to pelleted food and were housed in open cages with standard enrichment, and daily monitoring in accordance with local institutional guidelines.

Special effort was made to minimize animal suffering and to reduce the number of animals used to what was deemed necessary, considering the variability of each model, for the objectives of each experiment in accordance with the 'three R's' (refine, reduce and replace).

## 3.2 ANIMALS

It is of particular importance to choose the right strain of animals for the model of injury meant to be studied. In the context of neonatal HI, a certain degree of susceptibility or resistance to brain injury was previously reported when using CD1 and 129Sv mice, respectively. As the degree of brain damage in C57BL/6 mice was correlated with increasing duration of hypoxia, thus making this strain intermediately sensitive to HI (Sheldon, Sedik, & Ferriero, 1998), we chose mice on the C57BL/6 background for this thesis.

Wild type mice were used in **Paper III** to study the effects of caffeine treatment after neonatal HI, whereas genetically modified mice on the C57BL/6 background were used for all other studies.

In **Paper II**, $Cx3cr1^{EYFP\text{-}CreER}$ mice, which express the Cre-ERT2 fusion protein and have resident microglia ($CX3CR1^{+}$) labelled with enhanced yellow fluorescence protein (EYFP), were crossed with $Rosa^{DTA}$ mice, which carry a loxP-flanked stop cassette associated with an attenuated diphtheria toxin (Lund et al., 2018). Mice with genotype $Cx3cr1^{CreER\text{-}EYFP+}/Rosa26^{DTA+/-}$ (called $DTA^{+}$, microglia-depleted mice) and $Cx3cr1^{CreER\text{-}EYFP+}/Rosa26^{DTA-/-}$ ($DTA^{-}$, control mice) were included in this study and tamoxifen was administered intraperitoneally (50 mg/kg) at P8 and P9 to both groups.

Lastly, $CX3CR1^{GFP}$ mice, where green fluorescent protein (GFP) labels resident microglia ($CX3CR1^{+}$), were crossed with $CCR2^{RFP}$ mice, where red fluorescent protein (RFP) labels peripheral monocytes ($Ly6C^{hi}CCR2^{+}CX3CR1^{-}$) to discriminate between two distinct populations of cells (Saederup et al., 2010). The resulting $CX3CR1^{GFP/+}/CCR2^{RFP/+}$ mice were used in **Paper I** to characterize the role of these two cell types during the post-HI inflammatory cascade, and in **Paper IV** to identify the adoptively transferred heterologous bone-marrow derived macrophages from autologous $GFP^{+}$ and $RFP^{+}$ cells.

## 3.3 *IN VIVO* MODEL OF NEONATAL HYPOXIA-ISCHEMIA

In order to perform an accurate reproduction of neonatal HI in mice, postnatal day 9-10 (P9-P10) pups of both sex were selected for the experiments. The choice of age was based on a previous study that reported, after comparing brain development of humans and rodents (specifically looking at neuroanatomy, cell proliferation, synaptogenesis, myelination, and inflammation), that the level of brain maturation in term newborn infants is equivalent to P7-P10 pups (Semple et al., 2013).

The most commonly used model for neonatal HI derives from the Levine technique (Levine & Klein, 1960), later modified by Rice and Vannucci (Rice, Vannucci, & Brierley, 1981) and adapted again by Sheldon and colleagues (Sheldon et al., 1998). This model consists of exposure to systemic hypoxia after unilateral common carotid ligation. The simple ligation procedure is not sufficient to create an ischemic event in experimental animals due to extensive collaterals. The addition of hypoxia leads to vessel vasodilation that, in combination with the decreased blood pressure, creates a reduced cerebral flow in the ipsilateral hemisphere, thereby inducing unilateral brain damage (Vannucci & Hagberg, 2004).

In this thesis, unilateral ligation (**Paper I**) or unilateral electrocoagulation at 8 Watt (**Papers II - IV**), of the right carotid artery was carried out via midline neck incision under isoflurane sedation and local anesthesia to minimize pain and distress. The surgery took approximately 3-5 minutes per mouse. Pups were next returned to their mother for feeding purposes and then subjected to hypoxia (10% $O_2$ in 90% $N_2$ at 36˚C) for 50 (**Papers I - II**) or 60 minutes (**Papers III - IV**). There was little mortality, no severe illness or need for early euthanasia. During sham operation the carotid artery was visualized and isolated but not occluded.

## 3.4 MICROGLIA DEPLETION MODELS

In the past few years many strategies, be they pharmacological or genetic, have been developed to efficiently deplete microglia. Administration of CSF-1R inhibitors were shown to successfully pass through the BBB and to deplete microglial cells without otherwise affecting the mice, but repopulation was evident soon after interruption of the treatment. As an alternative, liposomal clodronate has been also implemented to deplete microglia, although it was reported to lead to alteration of mice social and learning behavior (J. Han, Harris, & Zhang, 2017).

A more specific option to the pharmacological depletion is the implementation of genetic models to ablate microglia. Examples include transgenic mice expressing the suicide gene herpes simplex virus thymidine kinase (HSVTK) under the CD11b promoter, via administration of the pro-drug ganciclovir, or CX3CR1CreER transgenic mice that drive diphtheria toxin receptor (DTR) expression upon Cre-mediated recombination, via administration of DTR. Both these models reach about 90% of microglia depletion, with a repopulation speed of 5 days (J. Han et al., 2017).

An improved model of the genetic depletion, which was used in **Paper II**, involves the implementation of Cx3cr1CreER mice crossed with $R26^{DTA}$ mice (Lund et al., 2018). This model is based on the CreER/lox system, where the DNA flanked between the loxP-specific sites is excised by CreER-recombinase, a ligand-dependent Cre-recombinase that is activated by administration of tamoxifen to the animals. This system leads to an almost complete depletion of the microglial niche (>95%).

## 3.5 BEHAVIORAL TESTS

HI induces brain damage in the hippocampus, striatum/thalamus, cerebral cortex and subcortical/periventricular white matter (Rice et al., 1981; Vannucci & Hagberg, 2004).
To identify any modulatory effect of our treatments, experimental mice underwent behavioral tests 2 weeks post-injury for the caffeine treatment (**Paper III**) project, or 3 weeks post-injury in the case of cell treatment (**Paper IV**). The tests were performed from a calm and unperfumed operator blinded to the treatment and with minimal handling to allow their natural behavior and to minimize stress.
Specifically, the following tests were used:
*Beam walk test*: this test is useful to evaluate their balance and fine movements (Goldstein & Davis, 1990). Mice are allowed to walk on a beam (0.7 cm wide, 60 cm long) elevated from the ground (50 cm high) and the number of mistakes is calculated (Aden et al., 2002).
*Open field*: this is a simple locomotor activity test that involves the observation (BIOBSERVE GmbH Software, St. Augustin, Germany) of the animal movements and explorative behavior within an open arena (50 cm per side) for 30 min.
*Rotarod*: this is a sensorimotor test that assesses coordination, where animals are placed on a rotating cylinder (Ugo Basile), left to walk for 300 seconds with increasing speed (4 to 40 rpm), and the latency calculated over an average of 5 trials.

## 3.6 *EX VIVO* MODEL OF HIPPOCAMPAL ORGANOTYPIC SLICES AND OXYGEN GLUCOSE DEPRIVATION

In order to reproduce the neonatal asphyxia *in vitro* we used an organotypic culture of hippocampal slices using the Stoppini technique (Stoppini, Buchs, & Muller, 1991). Briefly, P6-P7 pups were decapitated, their brains dissected and sliced at 300 μm, and the hippocampi were isolated and cultured on membrane inserts for up to a week. The choice of the mice age is based on the high degree of plasticity of the tissue that can therefore resist the mechanical trauma during slice preparation (Q. Li, Han, & Wang, 2016).

To mimic the effects of hypoxia-ischemia and reperfusion injury in slices, they are subjected to oxygen-glucose deprivation because effects such as cell death, excitotoxicity, and oxidative stress are greatly simulated in the *in vivo* microenvironment (Q. Li et al., 2016).
As slices can also be co-cultured with other cells it is also a useful tool to assess therapeutic effects. In **Paper IV,** M0 or M2 macrophages were co-cultured after OGD.

This *ex vivo* model has multiple advantages as it produces results in a short time period and in a precisely controlled system, preserving the three-dimensional cellular network. Organotypic slices are then to be considered a faster method than *in vivo* study, as it is possible to assess also specific pathways, and a more accurate method than the *in vitro* one, as the heterogeneity of the tissue is maintained.

## 3.7 TRANSCRIPTOMICS

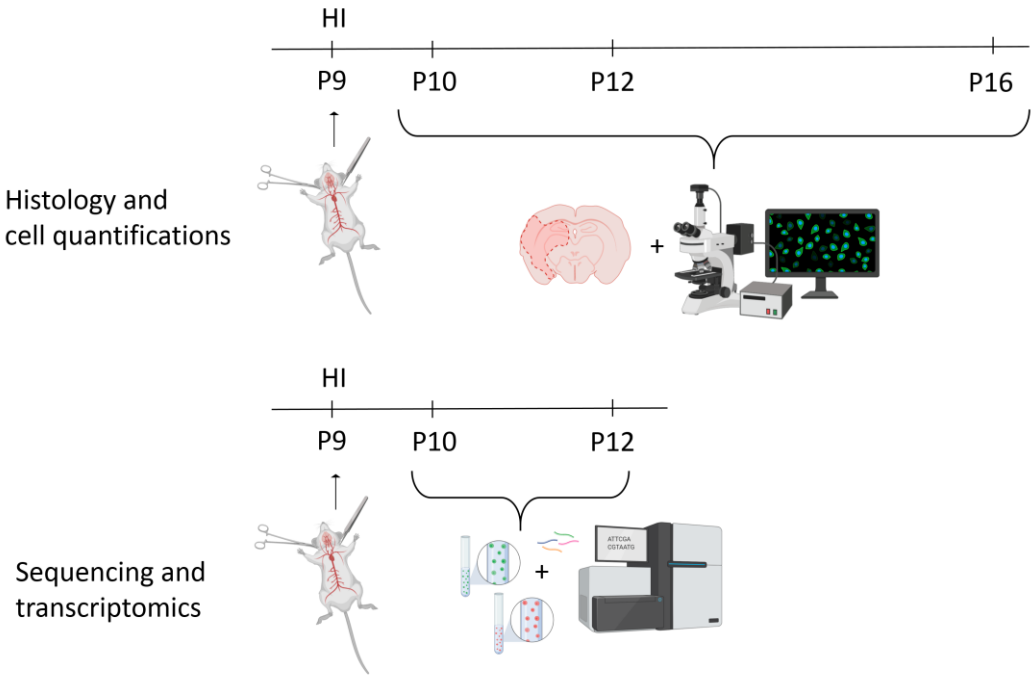
During the past decade the interest in omics studies has been the center of scientific research, and new generations of techniques have been implemented to define the whole genome and transcriptome (Stark, Grzelak, & Hadfield, 2019). RNA sequencing has been extensively used in biological and biomedical fields to study the mechanisms of complex diseases, to determine different cell population phenotypes or to identify biomarkers for clinical purposes (Wang et al., 2020).

In this thesis, we used bulk RNA sequencing to determine differentially expressed genes (DEGs) and signature markers between $GFP^+$ resident microglia and $RFP^+$ infiltrating macrophages. In order to do so, these two populations of cells were isolated using FACS sorting, RNA was extracted and converted into cDNA that was then sequenced. To gain an estimate of the expression level of each gene, cDNA fragments were aligned and mapped to a known transcriptome base.
More detailed information about the analysis can be found in **Paper I**.

# 4 RESULTS AND DISCUSSION

## PAPER I: SEX-DEPENDENT AND -INDEPENDENT INFLAMMATORY PATTERNS IN RESIDENT MICROGLIA AND INFILTRATING MACROPHAGES AFTER NEONATAL ASPHYXIA.

*Experimental design:*




*Aim:*

To study the interplay between resident microglia and infiltrating macrophages in the hippocampus after HI, and to determine if there is a sex-specific variability

*Background:*

Upon brain injury peripheral macrophages that infiltrate the brain parenchyma express a variety of common markers with resident microglia, thus making it difficult to identify the specific role of these two distinct populations of cells following HI.

*Hypothesis:*

Resident microglia and infiltrating macrophages have different functions during the post-ischemic neuroinflammation, and there are sex-specific differences.

*Results:*

$RFP^+$ cells infiltrate the hippocampus peaking 1 day after injury and decrease over time, being almost absent after a week. $GFP^+$ cells have a slow start and peak 3 days post-HI, keeping high density till 7 days after injury. There is no sex-specific difference in the cell densities. Transcriptomic analysis shows that $RFP^+$ cells have the greatest upregulation of inflammatory genes at the 1-day time point compared to the $GFP^+$ cells or the 3-day time point. In addition, males $GFP^+$ cells at the 3-day time point show a close clustering with control animals than do injured animals concerning microglia-unique genes.

In **Paper I** we investigated the role of resident microglia and infiltrating macrophages after HI. We used CX3CR1$^{GFP/+}$/CCR2$^{RFP/+}$ transgenic mice to selectively identify these two populations of cells. We started by reproducing similar injuries in both male and female mice, in order to have similar starting conditions, and we quantified the number of GFP$^+$ and RFP$^+$ cells in the ipsilateral hippocampus at different time points after HI. We saw that RFP$^+$ cells peaked 1 day after injury and that their numbers slowly decreased over time, whereas GFP$^+$ cells significantly increased 1 day post injury, peaking 3 days after HI and maintaining a plateau level until the 1 week time point. We did not observe a sex-specific difference in peripheral macrophage infiltration, in contrast to previous findings (Mirza, Ritzel, Xu, McCullough, & Liu, 2015; Villapol et al., 2019), nor did we detect sexual dimorphism in microglial cell density in either controls or injured mice (Guneykaya et al., 2018; Lenz & McCarthy, 2015; Schwarz et al., 2012).

Given the small number of RFP$^+$ macrophages infiltrating a week after HI, we decided to proceed by further analyzing these two cell types only at the 1 and 3 day time points. GFP$^+$ and RFP$^+$ cells were sorted separately from injured or control animals, and quantitative transcriptomic analysis was performed using RNA Smart-Seq technique (Picelli et al., 2014). We then analyzed bulk RNA-sequencing specifically looking into time, cell-type and sex parameters, and including the following groups: GFP-1d, GFP-3d, RFP-1d, RFP-3d, control-1d (GFP) and control-3d (GFP). An unsupervised analysis of the samples revealed that the two controls cluster together, while RFP and GFP samples showed specific differences over time. In detail, both the cell types were more diverse from the controls 1 day than 3 days after injury.

To better characterize this temporal pattern of GFP$^+$ and RFP$^+$ cells and to understand what are their specific roles in the post-ischemic inflammation, we next focused on the differentially expressed genes (DEGs, $q<0.05$) (Figure 2C). Analysis of the top 50 most-significant DEGs was then performed. An upregulation of apoptotic signaling genes 1 day after HI was observed in GFP$^+$ cells, whereas at the 3 day time point the same samples had an upregulation of genes in cell cycle, lipid metabolism and reorganization of synaptic activity. Similarly, similar analysis in RFP samples revealed that genes involved in inflammation, immune-response signaling, cell adhesion, chemotaxis, and extracellular matrix organization were upregulated after 1 day, while a clear upregulation of genes involved in lipid metabolism and transport was observed after 3 days.

As HI brain injury leads to a variety of endogenous inflammatory signals that promote either pro- or anti-inflammatory phenotypes at different stages of disease progression (Hellstrom Erkenstam et al., 2016; P. L. P. Smith et al., 2018), we analyzed the expression of genes related to M1 or M2 activation phenotypes. Interestingly, we observed that infiltrating macrophages expressed higher upregulation of genes belonging to both polarization states indicating that, upon entry to the brain, these RFP$^+$ cells rapidly activate and become the drivers of the inflammatory cascade.

Since the production of signaling molecules is highly dependent on the inflammatory environment (Colonna & Butovsky, 2017; Harris, 2014; Hickman et al., 2013), we analyzed specific families of genes including chemokines, cytokines, PRR and sensome markers. Similarly to the previous analysis, the RFP group had the highest upregulation of genes

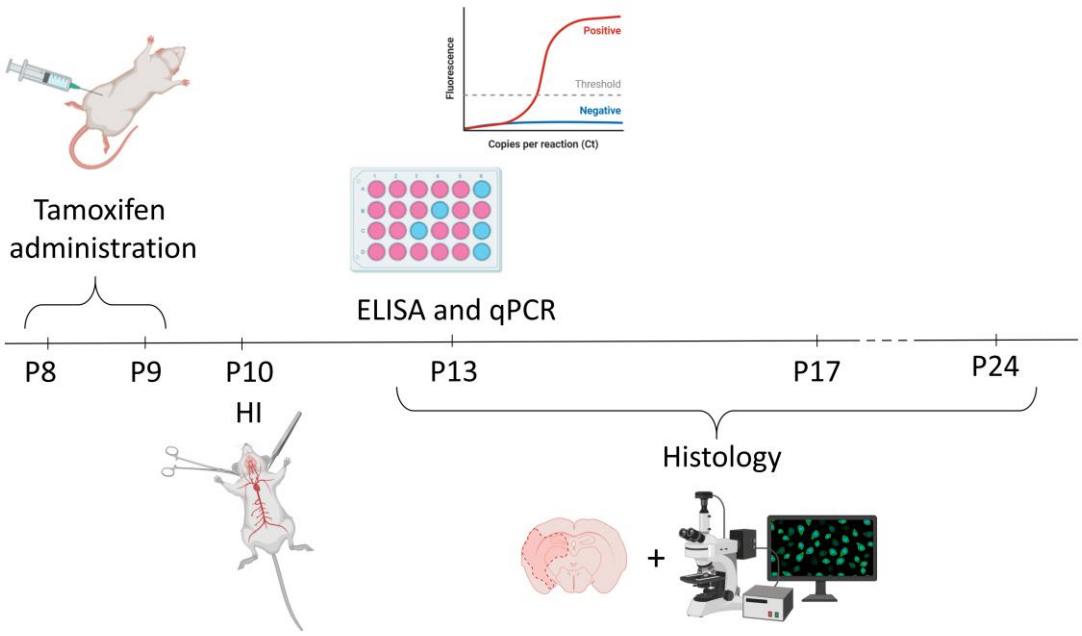
belonging to the PRR and sensome families 1 day after injury, highlighting the reactivity of these cells during post-ischemic inflammation. In addition, we observed a specific upregulation of chemokine receptors in the RFP-1d group, and a higher expression of cytokines and chemokines genes in the GFP-1d group. As previous studies have reported a sexual dimorphism after neonatal HI, in both the acute (Mirza et al., 2015) and chronic (Al Mamun, Yu, Romana, & Liu, 2018) phases of inflammation, we analyzed the DEGs in the groups in question. Sex-related differences were not observed 1 day after HI, but were at the 3-day time point. Among the DEGs, *Cx3cr1* and *Siglech* genes, which are also considered microglia-unique markers, were more upregulated in males than in females in both cell types.

We therefore proceeded by analyzing microglia signature genes. It is known that upon inflammation microglia downregulate unique genes, and it is also known that $RFP^+$ cells do not express these genes (Butovsky et al., 2014; Buttgereit et al., 2016; Krasemann et al., 2017). In accordance with the literature we observed that microglial signature genes were upregulated in control groups and downregulated in the RFP groups. As expected, such genes were downregulated in the GFP samples 1 day post-HI due to the activation of microglial cells during inflammation and, to a great extent these markers were also downregulated in the female GFP samples 3 days after HI. Surprisingly, the expression of the microglia-unique genes in the male GFP samples was almost at the level of controls at the 3-day time point. The UMAP clustering confirmed these findings, with the $GFP^+$ cells from males clustering with the controls 3 days after HI.

Emerging evidence of sexual dimorphism in microglia (Schwarz et al., 2012) and microglia responses to specific environments (Thion et al., 2018) suggest a different regulatory mechanism in males and females that may affect the post-ischemic inflammatory cascade and the consequent infiltration of immune cells. It is known that microglial metabolic mechanisms are of fundamental importance to their 'surveying' functions under physiological conditions, and that disturbance in this equilibrium can be associated with changes in microglial lipidome and pro-inflammatory phenotype (Chausse, Kakimoto, & Kann, 2020). Therefore, further analyses are needed to evaluate how HI brain injury affects the microglia lipidome and consequently the post-ischemic inflammation, and whether there are sex-specific differences.

## PAPER 2: AGGRAVATED BRAIN INJURY AFTER NEONATAL HYPOXIC ISCHEMIA IN MICROGLIA-DEPLETED MICE

*Experimental design:*




*Aim:*

To study the effects the effects of HI brain injury in a model of tamoxifen-based microglia-depletion.

*Background:*

Microglia are the brain phagocytes that scan and control the environment during physiological states. Upon injury, they undergo a structural change and they engulf cell debris and orchestrate the post-ischemic inflammation.

*Hypothesis:*

Microglia have a protective role during neonatal HI and depletion exacerbates the outcomes.

*Results:*

Tamoxifen administration at P8-9 resulted in >99% of microglia depletion in $DTA^+$ mice that persisted with 97% at P13. HI brain injury showed no difference in lesion extent between $DTA^+$ and $DTA^-$ female mice, but $DTA^+$ male mice had a significantly larger infarct volume than did $DTA^-$ male mice. $DTA^+$ mice of both sexes had a higher density of apoptotic cells in different brain regions compared to their respective $DTA^-$ mice. In addition, ELISA analysis revealed that $DTA^+$ mice of both sexes had significantly lower IL-10 and TGF-β levels in both sham and HI conditions, if compared to their respective $DTA^-$ mice.

In **Paper II** we studied the effects of HI brain injury in a novel model of microglia depletion. In the past few years, the role of microglia has been broadly investigated using several methods to deplete these cells (J. Han et al., 2017). The most common ones use pharmacological agents to ablate microglia. However, due to the time needed to reach an acceptable level of depletion, or the unselective cell damage, these models are not suitable for neonatal studies (Faustino et al., 2011; X. Han et al., 2019). The Harris lab has recently developed a genetic microglial-depletion model crossing $Cx3cr1^{EYFP\text{-}CreER}$ mice with $Rosa^{DTA}$ mice (Lund et al., 2018) based on tamoxifen administration. We optimized the dose (50 mg/kg) and timing (P8 and P9) for tamoxifen administration in mice with either $Cx3cr1^{CreER\text{-}EYFP+}/Rosa26^{DTA+/-}$ (called $DTA^+$, microglia-depleted mice) or $Cx3cr1^{CreER\text{-}EYFP+}/Rosa26^{DTA-/-}$ ($DTA^-$, control mice) genotypes. This protocol induced > 99% microglia depletion, as judged by $Iba1^+$ positive cell counting, by P10 and this was maintained quite stably until P13 with a rate of depletion > 97%, making it a suitable model to study the effects of HI. Microglia repopulation then proceeded faster, with a rate of 60-70% depending on the brain region by P17 where non-specific clustering of repopulating cells was observed. Complete repopulation was observed at P24 with a slightly higher number of microglial cells in the $DTA^+$ mice. Such overpopulation was consistent with previous reports of pharmacological depletion (Huang et al., 2018).

HI was performed at P10 in animals belonging to both groups and both sexes and, given the variability of repopulation at P17, we assessed changes in microglial proliferation and injury progression at P13. Using flow cytometry we analyzed $EYFP^+$ microglia in the ipsi- and contra-lateral hemispheres of injured mice, as well as in sham animals. We did not observe any difference in the repopulation rate after injury, and this was also confirmed with immunohistochemical analysis for the EYFP marker.

We then proceeded evaluating the effect of microglia depletion on HI brain injury. By contouring MAP2 unstained tissue we found that $DTA^+$ males had a larger lesion compared to $DTA^-$ males, and that $DTA^+$ males had a larger lesion compared to $DTA^+$ females. Similar findings were also observed in a model of adult stroke after pharmacological depletion (Faustino et al., 2011). This sexual dimorphism in the injury size after microglial ablation could be also related to specific characteristics of microglial cells, such as the ability to migrate faster in males than in females (Schwarz et al., 2012; Weinhard et al., 2018; Yanguas-Casás et al., 2018) and this may have protective effects in non-depleted mice.

Next, we quantified neuronal TUNEL-related apoptosis and we observed that the majority of $TUNEL^+$ cells clustered in the NeuN unstained area, which overlapped with MAP2 unstained tissue. $DTA^+$ males had a higher number of $TUNEL^+$ cells compared to $DTA^-$ males in the cerebral cortex, thalamus and caudoputamen. Similarly, $DTA^+$ females had a higher number of $TUNEL^+$ cells compared to $DTA^-$ females in the hippocampus and thalamus. When sexes were compared we observed a tendency towards exacerbated apoptosis in males compared to in females in most areas of interest. Such differences seem to be associated with mitochondrial metabolism and apoptotic mechanisms previously reported to differ between sexes in physiological and pathological conditions (Demarest, Schuh, Waddell, McKenna, & Fiskum, 2016; Weis et al., 2014; Zhu et al., 2006).

Finally, we performed gene expression analysis to define whether microglia depletion had an effect on cytokine and chemokine production at P13, following HI at P10. Our results revealed a reduced expression of *IL10* and *TGFβ* genes in $DTA^+$ males compared to in $DTA^-$ males, while among females only *TGFβ* had a reduced expression. However, no difference was observed at P10, indicating a similar starting condition for all mice. Our findings are quite

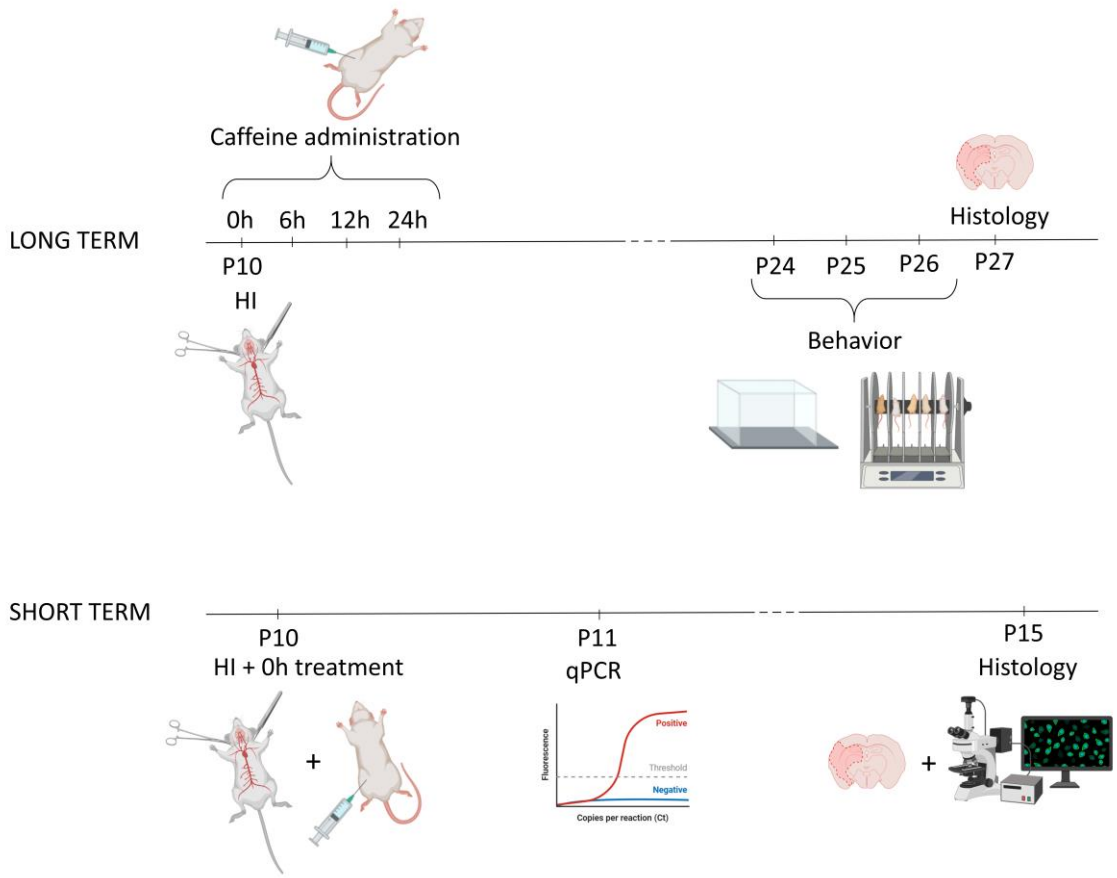
moderate compared to previous studies in which adult stroke in microglia-depleted animals led to significantly increased levels of pro-inflammatory cytokines (Jin et al., 2017). However, this could be due to the fact that we assessed gene expression during a sub-acute phase of injury.

Based on the outcomes of the gene expression analyses we measured protein levels. $DTA^+$ mice of both sexes had lower levels of IL-10 and TGFβ compared to $DTA^-$ mice. In addition, an increase of TGFβ was observed in injured $DTA^+$ mice of both sexes compared to shams, while only male $DTA^-$ mice had higher levels of TGFβ compared to shams. These findings suggest that microglia have a fundamental role in producing IL-10 in mice, as depletion leads to undetected levels of this cytokine. In addition, microglia were shown to be the major source of TGFβ after stroke (Lehrmann et al., 1998), although other cells were reported to produce it after microglia depletion (Recasens, Shrivastava, Almolda, González, & Castellano, 2019).

Taken together, the results show that this genetic model for microglia depletion is a useful tool to further characterize the function of microglial cells in physiological and pathological contexts in the neonatal brain. Given the sexual dimorphism in both sham and HI mice following microglia depletion, and the unknown effects on specific inflammatory or injury-related mechanisms, further studies aimed at assessing astrocytic involvement in this model are needed.

## PAPER 3: DEFINING A TIME WINDOW FOR NEUROPROTECTION AND GLIA MODULATION BY CAFFEINE AFTER NEONATAL HYPOXIA-ISCHAEMIA.

*Experimental design:*




*Aim:*

To study whether caffeine exerted neuroprotection after HI and the duration of the therapeutic time window.

*Background:*

Caffeine is a competitive antagonist of the adenosine receptor. It is commonly used in the clinic to treat neonatal apnea and long-term follow-up in apneic children showed a reduced incidence of cerebral palsy.

*Hypothesis:*

Caffeine has a protective role if administered after neonatal HI.

*Results:*

Caffeine induces functional recovery and neuroprotection only when administered directly after HI. Later treatments do not have an effect. Caffeine reduces the number of amoeboid microglia and apoptotic cells, limits glial scar formation and white matter damage, and downregulates inflammatory genes after HI.

In **Paper III** we studied the effects of caffeine treatment after HI brain injury. A previous study from the Ådén group demonstrated that caffeine can offer neuroprotection and functional recovery in a mild form of neonatal HI (Winerdal et al., 2017). We therefore aimed to assess whether caffeine exerted the same effects in a more severe model of injury, and to define the caffeine therapeutic time window.

Specifically, we administered a single dose of 5mg/kg caffeine at 0, 6, 12 and 24 hours after HI, which was induced at P10, and performed behavioral studies starting from P21. Our results showed that while caffeine did not improve coordination in rotarod tests, it restored the behavioral deficits induced by HI in the open field test when administered acutely (0h). The animal activity during the test was indeed normalized to the level of the controls, suggesting habituation to the arena only at that time point.

Histological analyses also confirmed the previously observed neuroprotective effect of caffeine. We evaluated the extent of global injury by assigning a neuropathological score to cresyl violet-stained sections, and we defined the percentage of tissue loss by contouring MAP2 unstained tissue. In both analyses the lesion was reduced only at the 0h time point, in concert with behavioral analyses. No difference was evident after later administrations.

Previous studies have reported amelioration in HI rodents after multiple injections or higher doses of caffeine (Alexander, Smith, Rosenkrantz, & Fitch, 2013; Potter, Rosenkrantz, & Fitch, 2018), but none of them had defined a therapeutic time window. In our study we therefore highlighted the importance of acutely administering caffeine after HI. Because no effect was observed when caffeine was administered at later time points, we decided to only proceed with further analysis with the 0 h treatment and we characterized the effects of caffeine 5 days after HI.

We observed that MAP2 tissue loss was reduced after caffeine injection, mainly in the hippocampus and striatum, suggesting that caffeine neuroprotective mechanisms involved both adenosine $A_1$ and $A_{2A}$ receptors. Indeed, adenosine $A_1$ receptor is highly expressed in the hippocampus while adenosine $A_{2A}$ receptor is highly expressed in the striatum (Cunha, 2005). Delineation of GFAP reactive astrocytosis revealed perfect overlap with MAP2 unstained tissue. Similarly to the MAP2 analysis, the caffeine-treated group had a reduced glial scar in the hippocampus and striatum, and presented a less extensive astrogliosis in the cortices, specifically between layers 2/3 and, less prominently, in layer 5. Although no comparable MAP2 injury was observed in the cortices to justify the reactivity of astrocytes, an elevated number of $TUNEL^+$ cells was instead associated with the extent of the glial scar. Since mice treated with caffeine had a lower number of $TUNEL^+$ cells, as supported by others (Kilicdag, Daglioglu, Erdogan, & Zorludemir, 2014), the consequent smaller glial scar could be self-explanatory. However, it was previously reported that caffeine selectively antagonizes adenosine $A_{2A}$ receptor in astrocytes, thereby affecting their immunoreactivity and proliferation (Desfrere et al., 2007), and this suggests a direct modulatory effect of caffeine.

To assess whether microglia were also modulated by a smaller lesion and reactive astrocytes, we performed Iba1 staining and analyzed cells with an amoeboid cell body. We determined that mice treated with caffeine had a lower number of amoeboid microglia in the striatum and cortices, and that the soma size of these cells was smaller than in the PBS-treated HI group. Recent findings demonstrated that caffeine can suppresses microglial production of pro-inflammatory mediators (Kang et al., 2012) that are associated with the M1 phenotype induced by adenosine (Colella et al., 2018). Similarly, activation of adenosine $A_{2A}$ receptor causes cytokines production and retraction of microglial processes, leading to the active state

(Minghetti et al., 2007; Orr, Orr, Li, Gross, & Traynelis, 2009) while blockade of this receptor reduces microglial activation (Rebola et al., 2011), also confirming caffeine modulatory effects on microglial cells.

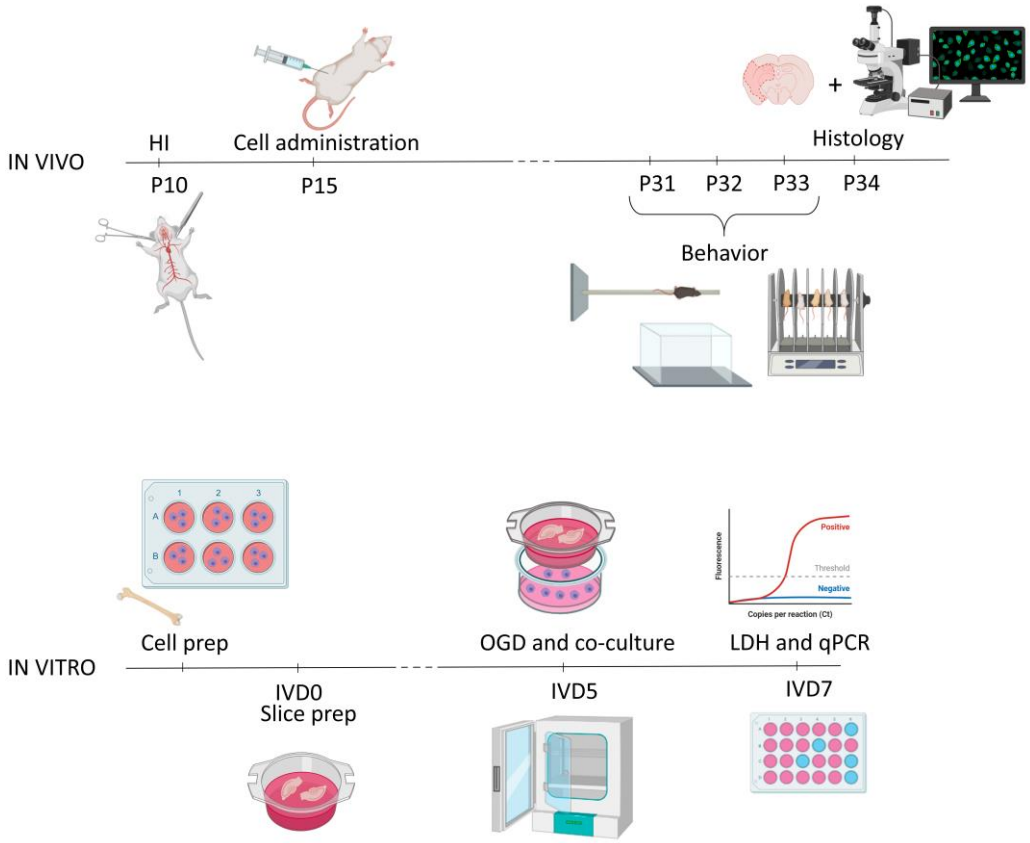
Finally we analyzed the white matter (WM) damage and we demonstrated that caffeine specifically preserved myelination in the striatum, where a higher concentration of $MBP^+$ fibers are detected. Hypoxia is known to alter the maturation of oligodendrocyte progenitors (OP) cells and thus affects myelination while conversely caffeine was shown to invert this process and to promote OP normal development by inhibiting the adenosine $A_1$ receptor (Cao et al., 2019; Othman, Yan, & Rivkees, 2003; Stevens, Porta, Haak, Gallo, & Fields, 2002). In support of our results, caffeine treatment was shown to enhance myelination in a model of periventricular WM injury (Back et al., 2006).

Caffeine binds to some extent to all adenosine receptors, with a preference for the adenosine $A_1$ and $A_{2A}$ receptors that are expressed in the pre- and post- synaptic site of neurons, but that are also present in microglia, astrocytes and oligodendrocytes (Sheth, Brito et al. 2014). By binding to ARs, caffeine thus modulated the cells during injury, as our study has demonstrated.

Taken together, these results show beneficial effects of caffeine in the form of functional recovery and neuroprotection, after a single administration. As caffeine is currently used in the clinic to teat apneic babies (Aranda, Collinge, Zinman, & Watters, 1979) (Johnson, 2011; Natarajan, Lulic-Botica, & Aranda, 2007) and long-term follow-up studies have shown a reduction in the incidence of cerebral palsy in treated children (Schmidt et al., 2007), we suggest that caffeine is a potential therapeutic agent also for neonatal asphyxia.

## PAPER 4: ADOPTIVE TRANSFER OF BONE-MARROW-DERIVED MACROPHAGES MODULATES POST-ISCHEMIC INFLAMMATION IN A MODEL OF NEONATAL HYPOXIA-ISCHEMIA.

*Experimental design:*




*Aim:*

To study the effects of an adoptive transfer of bone marrow-derived macrophages in different polarization states after HI.

*Background:*

Previous studies have investigated the therapeutic potential of different cells types, including monocytes, to treat neonatal HI. Macrophages have different polarization states depending on the signals they receive at the microenvironmental level, and are capable of immunomodulary activities.

*Hypothesis:*

Polarization of the cells used as treatment influences the injury outcomes.

*Results:*

Peripheral macrophages peak in the ipsilateral hemisphere 1 and 7 days after HI. Adoptive transfer of BMDMs differentially modulated the injury outcomes depending on the administered phenotype. M0 macrophages led to worsening of the behavioral performance, whereas M2 macrophages promoted functional recovery. Our *in vitro* results showed that M0 cells co-cultured with hippocampal organotypic slices subjected to OGD had an upregulation of pro-inflammatory genes, thus suggesting induction to an M1 phenotype. On the contrary, M2 cells maintained their anti-inflammatory state.

In **Paper IV** we studied the infiltration of peripheral macrophages in the injured brain after HI. We identified a biphasic pattern whereby $RFP^+$ cells peak in the ipsilateral hemisphere after 1 day and 7 days in injured animals. It is well known that immune cells reach the injury site shortly after HI and that their role is fundamental in the post-ischemic neuroinflammation (Hagberg et al., 2015; Lai et al., 2017). In accordance with previous studies reporting that infiltration of immune cells protracts in time (Bona et al., 1999; P. L. P. Smith et al., 2018; Winerdal et al., 2012), we also observed that the number of $RFP^+$ cells in the ipsilateral hemisphere was higher than the controls up to 14 days post-HI. Increasing evidence of the role of chronic post-ischemic inflammation suggests that targeting this tertiary phase of disease with immunomodulatory therapies could promote neuroprotection and lead to tissue repair (Davidson et al., 2018; Fleiss & Gressens, 2012; Gliem et al., 2012).

Tissue-reparative processes indeed rely on factors such as resolution of inflammation and healing, and macrophages, which play a crucial role during HI inflammation, are capable of producing anti-inflammatory cytokines/chemokines and eliminating tissue debris (Harris, 2014). The Harris lab previously demonstrated that simultaneous stimulation with IL-4/IL-10/TGFβ could induced a stable anti-inflammatory phenotype (M2) in BMDMs and how, upon administration in diseased animals, such cells significantly modulated the disease progression in Type 1 diabetes (Parsa et al., 2012) and multiple sclerosis (X. M. Zhang, Lund, Mia, Parsa, & Harris, 2014) models.

Given the extensive recruitment of macrophages in our HI injury model, and therefore the extended time frame for a cell-based therapeutic approach, we decided to stimulate BMDMs *ex vivo* into an M2 phenotype and to inject these cells during the sub-chronic phase of HI inflammation. This approach was quite novel since no other study had so far evaluated the therapeutic potential of M2 cells in neonatal models of injury, although others have previously evaluated the effects of different sources of mononuclear cells as treatment for perinatal asphyxia (Sato et al., 2018; J. Zhang et al., 2019).

In our experimental design HI was performed at P10, and M2 or M0 cells were injected i.p at P15 and behavioral testing was performed between P31-P34. The time point for cell administration was chosen considering the infiltration window of peripheral macrophages. In addition, in order to distinguish between the effect of the cell type and the effect of the specific phenotype, we decided to use M0 cells as controls. This choice was of fundamental importance as we observed different outcomes depending on the type of cells administered.

The beam walk test showed that when mice received M2 macrophages after HI they performed better, making fewer mistakes than injured mice receiving PBS, indicating improvements in balance and fine movements. Conversely, worsening of the performance was observed when M0 macrophages were administered. Although we expected rotarod performance to concord with the beam walk, our results did not show any statistical difference in the treated mice. Injured animals performed worse than did shams, and M0 cells similarly led to a trend towards a poorer coordination performance compared to the PBS-treated mice. However, no improvement was observed in animals receiving M2. This is not the first case in which various behavioral tests assessing similar functions have resulted in different outcomes. Interestingly, findings similar to ours were previously observed in a study in which HI rats treated with bone marrow-derived mononuclear cells had a better performance in the gait-CatWalk test, indicating an improved coordination, but no differences were observed in the rotarod test (Sato et al., 2018).

Histological analysis aimed at assessing the extent of the lesion were partially in line with the behavioral tests. Cresyl violet-stained sections were used to assess the global injury using the NPS system, while tissue atrophy was measured by contouring unstained MAP-2 tissue. We determined that mice receiving M0 macrophages had a trend towards a larger injury and an increased tissue loss compared to HI mice treated with PBS. Surprisingly, no difference was observed in animals that received M2 macrophages. It was previously reported that, although human cord blood-derived mononuclear cells significantly reduced motor deficits in a model of neonatal HI, they failed to offer neuroprotection (Meier et al., 2006).

Neuroprotection may not be the modulatory role of M2 macrophages in our experimental design. Although M2 cells are generally associated with reduction of tissue damage, increased cell survival, and promotion of wound healing (Dey, Allen, & Hankey-Giblin, 2014; Mantovani et al., 2004), we hypothesize that they may have other functions in this specific context. Cross-talk between astrocyte proliferation and monocyte invasion was previously reported to reduce glial scarring in brain injury (Frik et al., 2018) and we suggest that M2 cells may thus have modulatory effects on astroglial scar formation.

Concerning the M0 cells, we instead hypothesize that, even though the treatment was performed during the sub-acute phase of HI, the injury microenvironment may have led to unpolarized macrophages shifting towards a pro-inflammatory phenotype. Macrophages are indeed known to have either harmful (M1) or healing (M2) roles depending on specific signals they receive in a specific context and at a specific disease stage (Harris 2014). Indeed, although the specific phenotypic memory of M2 cells seem to be maintained even when exposed to a highly pro-inflammatory environment, the same cannot be said for M0 cells (Italiani & Boraschi, 2014; Viola, Munari, Sánchez-Rodríguez, Scolaro, & Castegna, 2019). We thus performed OGD in organotypic hippocampal slices and co-cultured them with either M0 or M2 cells for 48h to assess the effect of a high cytotoxic environment on macrophages. Our results indicated a higher expression of pro-inflammatory genes in M0 cells, thus suggesting induction of a pro-inflammatory phenotype. In contrast, M2 cells were capable of maintaining their identity, confirming previous reports (Parsa et al., 2012).

Taken together, these results confirm that macrophages have a fundamental role in the post-ischemic inflammation, as supported by the fact that depletion of macrophages after stroke leads to increase injury (Gliem et al., 2012; Wattananit et al., 2016), and that macrophages might have a successful therapeutic value only if they are in the M2 phenotype, as supported by previous studies on functional recovery in the central nervous system (Ma et al., 2015).

# 5 CONCLUSIONS

**Paper I.**

- Infiltrating macrophages peaked one day after injury and slowly decrease over time, whereas resident microglial numbers significantly increase one day and peak three days after HI.
- Infiltrating macrophages were the drivers of the inflammatory cascade by expressing the majority of upregulated markers in the M1/M2, chemockines/cytokines, PRR and sensome families of genes.
- Sexual dimorphism was observed three days after injury in both $GFP^+$ and $RFP^+$ cells
- Downregulation of homeostatic genes was observed one day after injury in both sexes, but only in females at the 3-day timepoint suggesting that male microglia return faster to control levels.

**Paper II.**

- The genetic model for microglia depletion obtained by crossing $Cx3cr1^{EYFP\text{-}CreER}$ mice with $Rosa^{DTA}$ mice led to 99% microglial ablation in neonatal mice.
- HI brain injury did not lead to faster repopulation.
- Resident microglia played a neuroprotective role early after neonatal hypoxic ischemia.
- Aggravating effect of microglia depletion was predominant in males and was associated with lower levels of anti-inflammatory cytokines.

**Paper III.**

- The therapeutic time window of caffeine was very short.
- A single dose of caffeine given acutely after HI led to:
    - reduced grey and white matter damage, and apoptotic cell density
    - decreased amoeboid microglia and the area of astrogliosis
    - modulation of the expression of pro-inflammatory cytokines
    - functional recovery in terms of restored open field activity

**Paper IV.**

- Peripheral macrophages infiltrated the CNS in a biphasic pattern, peaking in the ipsilateral hemisphere one and seven days after HI.
- Adoptive transfer of M0 macrophages led to worsening of the behavioral performance and a tendency towards a larger injury as they shifted toward a pro-inflammatory phenotype.
- Adoptive transfer of M2 macrophages promoted functional recovery by maintaining their anti-inflammatory state even after being exposed to a second inflammatory stimulus.

# 6 FUTURE PERSPECTIVES

In my thesis we demonstrated that microglial cells have a protective role in the neonatal brain, that peripheral macrophages are the main players in post-ischemic inflammation, and that caffeine and M2 cells should be further investigated as potential therapeutic agents for HI brain injury.
Based on our results, I suggest the following research questions to guide future experiments.

**Do resident microglia mature differently in males and females?**
As we demonstrated in **Study I**, microglial cells deriving from injured male hippocampi had an upregulation of homeostatic genes 3 days after HI compared to females. This sexual dimorphysm in the expression of signature genes suggests a different mechanistic response of microglial cells to brian injury. As clinical evidence and pre-clinical studies reported that males have a higher incidence of worse long-term outcomes in injuries associated with the perinatal period (Wood et al., 2005), further investigations are needed to determine whether this difference may be associated with microglia maturation, and if so whether it is causative (males are more affected because microglia matures differently) or consequential (microglia maturation in males is affected due to HI). A possibille analysis to determine microglial maturation could be the identification of the Microglia Developmental Index (MDI) as recently described by Hanamsagar and colleagues (Hanamsagar et al., 2017). By sequencing microglial cells from embryonic till adult mice the authors identified specific genes expressed in different developmental stages that could define an index of microglia maturation. Applying this MDI to human transcriptome data, they recorded disease-specific microglia development alterations and similarities with reported clinical studies concerning disease incidence. Thus, by analyzing the MDI in our data we could therefore deepen our understanding of the role of microglia in HI brain injury.

**What drives the post-ischemic inflammation after microglia depletion?**
In **Study II** we demonstrated that microglial depletion before HI brain injury leads to a larger infarction size and a higher number of apoptotic cells than in control mice. However, it is yet to be defined how cell debris is removed and inflammatory molecules to guide the post-ischemic inflammation are produced when microglia are depleted. In addition, the role of other glial cells and infiltrating immune cells has not been investigated.

Recent findings have demonstrated that peripheral monocytes can colonize the adult brain after microglia depletion and acquire a microglial-like phenotype although maintaining a distinct transcriptional, functional and epigenetical diversity (Lund et al., 2018). This raises the question as to the origin of repopulating microglial cells in the neonatal brain, and the specific role of infiltrating macrophages when microglia are depleted.

**How do adoptively transferred macrophages lead to functional recovery?**
In **Study IV** we demonstrated that an adoptive transfer of M2 macrophages given at P15 improves the behavioral performance of animals 3 weeks after HI. However, no difference in the lesion extent was observed between mice that received vehicle or cell treatment. Recent findings have shown a correlation between the extent of the astrocytic glial scar and the rate of infiltration of peripheral monocytes (Frik et al., 2018). The latter were reported to actively

modulate the composition of the extracellular matrix and the consequent formation of scarring tissue after traumatic-brain injury (Kjell & Götz, 2020). Since it is known that the glial scar is part of the tertiary phase of HI brain injury, we hypothesize that the modulatory effects of M2 cells may be directly related to astrocytes.

In general a lot can be learned from preclinical studies, but the road from rodent experiments to clinical practice is quite long. Therefore the next step towards identifying targets and specific mechanisms of recovery that may be exploited for therapeutic development would be to implement larger animal models. Although challenging, such an effort will aid in addressing the gap in translating bench science to bedside treatments.

Moreover, additional studies should be performed to evaluate the therapeutic potential of mononuclear cells derived from other sources, such as umbilical cord blood (UCB), differentiated into M2 macrophages. UCB is readily available at birth, and it could result in autologous treatment, thus reducing immunoreactivity risks of heterologous administrations (Castillo-Melendez et al., 2013). However no previous studies have evaluated the effects of M2 cells deriving from UBC.

# 7 AKNOWLEDGEMENTS

It is unbelievable to think that I am almost done, considering that so much has happened during this PhD…a massive water leak, three lab-moves, and a world pandemic?!? This journey has finally reached an end and I wouldn't be here if it wasn't for my supervisors, colleagues, friends and family, and I owe them big thanks for their support!

I am truly grateful to my supervisor **Ulrika Ådén** for giving me this opportunity. I remember our first meeting, during the selection process to fill the PhD position. You asked me something like "where do you see yourself in the future?" and I answered "in your chair, or better, not yours but another one, as group leader". I was young and determined, and you were (are) many things: a woman, a doctor, a mother, a researcher, a musician…plenty to take inspiration from! Thank you for giving me independence and supporting me to develop as an independent researcher.

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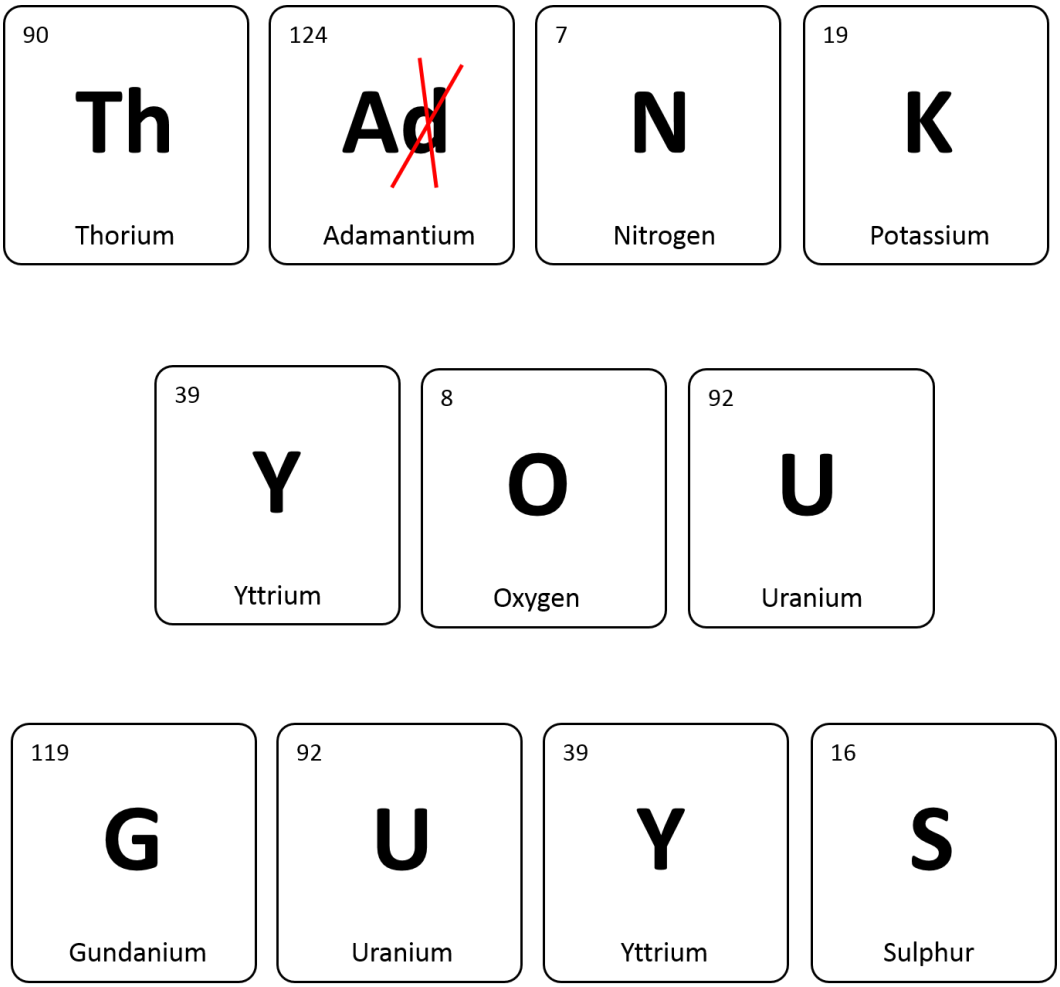
I hope I haven't forgotten anyone, and if I did I hope I can be forgiven. This section has actually been the hardest one for me to write because (those who know me well are fully aware of it) I am a very, very, very emotional person!
I leave you all with this wonderful and nerdy *thank you* that my lovely husband has made for me. Yes, it does include fictional elements too :-)



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